Developmental Biology 344 (2010) 992–1000

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

The PAM-1 aminopeptidase regulates centrosome positioning to ensure anterior–posterior axis specification in one-cell *C. elegans* embryos

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ARTICLE INFO

Article history:
Received for publication 12 May 2010
Revised 9 June 2010
Accepted 11 June 2010
Available online 19 June 2010

Keywords:
Puromycin-sensitive aminopeptidase
Polarity
C. elegans
PAR proteins
Centrosome

ABSTRACT

In the one-cell *Caenorhabditis elegans* embryo, the anterior–posterior (A–P) axis is established when the sperm donated centrosome contacts the posterior cortex. While this contact appears to be essential for axis polarization, little is known about the mechanisms governing centrosome positioning during this process. *pam-1* encodes a puromycin sensitive aminopeptidase that regulates centrosome positioning in the early embryo. Previously we showed that *pam-1* mutants fail to polarize the A–P axis. Here we show that PAM-1 can be found in mature sperm and in cytoplasm throughout early embryogenesis where it concentrates around mitotic centrosomes and chromosomes. We provide further evidence that PAM-1 acts early in the polarization process by showing that PAR-1 and PAR-6 do not localize appropriately in *pam-1* mutants. Additionally, we tested the hypothesis that PAM-1's role in polarity establishment is to ensure centrosome contact with the posterior cortex. We inactivated the microtubule motor dynein, DHC-1, in *pam-1* mutants, in an attempt to prevent centrosome movement from the cortex and restore anterior–posterior polarity. When this was done, the aberrant centrosome movements of *pam-1* mutants were not observed and anterior–posterior polarity was properly established, with proper localization of cortical and cytoplasmic determinants. We conclude that PAM-1's role in axis polarization is to prevent premature movement of the centrosome from the posterior cortex, ensuring proper axis establishment in the embryo.

Introduction

The anterior–posterior axis in *Caenorhabditis elegans* is established at the one-cell stage (reviewed in Lyczak, 2007). Sperm entry into the egg triggers completion of oocyte meiosis (McCarter et al., 1999; McNally and McNally, 2005) and around the time of meiotic exit, components donated by the sperm act to polarize the axis (reviewed in Munro and Bowerman, 2009). Work from many groups has shown that the sperm donated centrosome plays a key role in this process (Cowan and Hyman, 2004; Hamill et al., 2002; O'Connell et al., 2000; Sadler and Shakes, 2000). The centrosome contacts the cortex and, through an unknown mechanism, contributes to the actomyosin destabilization in the posterior and consequent cortical cytoplasmic flows to the anterior (Munro et al., 2004). These flows require inhibition of Rho activity in the posterior, which is controlled through depletion of the Rho-GEF, ECT-2, from the posterior, and local activity of the sperm donated Rho-GAP, CYK-4 (Jenkins et al., 2006; Motegi and Sugimoto, 2006; Schonegg and Hyman, 2006). This results in enhanced myosin contractility in the anterior as compared to the posterior (reviewed in Munro and Bowerman, 2009). The differences in contractility at the two poles results in formation of a pseudocleavage furrow between the two cortical domains and flows of cortical cytoplasm to the anterior pole (Goldstein and Hird, 1996; Hird and White, 1993). These cortical flows result in localization of the polarity determinants PAR-3, PAR-6 and PKC-3 to the anterior cortex (Cuenca et al., 2003; Goldstein and Hird, 1996; Hird and White, 1993; Jenkins et al., 2006; Munro et al., 2004). This event then allows PAR-2 and PAR-1 to localize to the posterior cortex (Cuenca et al., 2003; Hao et al., 2006; Munro et al., 2004). The localization of the PAR proteins to distinct domains on the cortex leads to later asymmetries in the embryo, such as an asymmetric first cleavage and localization of cytoplasmic determinants to the two poles (reviewed in Lyczak, 2007). For example, the germ-line P granules and the cytoplasmic PIE-1 protein are segregated to the posterior pole prior to the first mitosis (Cheeks et al., 2004; Hird et al., 1996; Strome, 1986; Tenenhaus et al., 1998). These asymmetries are key for establishing the axis and ensuring that the two daughter cells of the first division have different fate potentials.

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doi:10.1016/j.ydbio.2010.06.016

Table 1
C. elegans strains used in this study.

Description	Strain	Genotype	Reference
pam-1 deletion	EU934	*pam-1*(or282) IV	Lyczak et al. (2006)
pam-1 nonsense	EU1019	*pam-1(or403) IV*	Lyczak et al. (2006)
Tubulin::GFP	AZ244	*unc-119(ed3) III; ruIs57[unc-119(+) pie-1::GFP::tubulin]*	Praitis et al. (2001)
pam-1; Tubulin::GFP	US27	*unc-119(ed3) III; pam-1(or403) IV; ruIs57[unc-119(+) pie-1::GFP::tubulin].*	this study
PAR-1::GFP	JH1848	*unc-119(ed3) III;axIs1327*	kindly provided by G. Seydoux
PAR-6::GFP	FT17	*xnIs3[par-6:PAR-6::GFP+unc-119(+)] unc-119(ed3) III*	Anderson et al. (2008)
pam-1; PAR-6::GFP	US22	*xnIs3 III; pam-1(or403)IV;*	this study
pam-1; PAR-1::GFP	US25/26	*pam-1(or403) IV; axIs 1327*	this study
PIE-1::GFP	JH1327	*axEx73 [pJH3.92]*	Reese et al. (2000)
pam-1; PIE-1::GFP	EU929	*pam-1(or282)/DnT1 IV; +/DnT1V;axEX73*	Lyczak et al. (2006)

Previously we identified mutations in *pam-1*, which encodes a puromycin sensitive aminopeptidase that is required for the establishment of anterior–posterior polarity (Lyczak et al., 2006). These aminopeptidases have been identified across a large range of species, and have been implicated in numerous cellular processes (reviewed in Thompson and Hersh, 2004) but little is known of their *in vivo* functions. We have shown that PAM-1 is required for timely exit from meiosis and establishment of anterior–posterior polarity in the early embryo (Lyczak et al., 2006). In many *pam-1* mutant embryos, pseudocleavage, cortical flows and asymmetric distribution of PAR proteins are absent. As a result, many *pam-1* mutant embryos divide symmetrically and lack all signs of axis polarization. The polarity defects are separable from the meiotic exit defects, since inactivation of the B-type cyclin CYB-3 can rescue the meiotic exit defect but not the polarity defect (Lyczak et al., 2006). How PAM-1 regulates polarity is unknown; however, previously we hypothesized that it does so by regulating the dynamics of the sperm-donated centrosomes. In *pam-1* mutants, the centrosomes spend a significantly shorter amount of time in contact with the posterior cortex. The centrosomes often move around in the cell prior to completion of meiosis and thus are not in the vicinity of the cortex to polarize the axis (Lyczak et al., 2006).

Normal movement of the centrosomes and the associated pronuclei requires the microtubule cytoskeleton. The dynein heavy chain motor DHC-1 and its regulator LIS-1 are both necessary in wild-type embryos for the centrosomes to move from the cortex after polarity establishment and for pronuclear migration and centration prior to the first mitotic division (Cockell et al., 2004; Gönczy et al., 1999). Similarly, the dynactin components DNC-1 and DNC-2 are necessary for these movements (Gönczy et al., 1999; Skop and White, 1998). The actions of these proteins must be coordinated with the cell cycle such that movements of the centrosomes occur only after meiosis is complete. While proteins necessary for the movements of the centrosomes after polarity establishment are known, factors necessary for positioning the centrosome during cell polarization have not yet been defined.

Here we provide further evidence that PAM-1 is necessary at the earliest step in axis polarization, for positioning of the centrosome. We show that PAM-1 is localized to the cytoplasm throughout early development, with enrichment near mitotic chromosomes and microtubules. We also examine the dynamics of PAR localization in *pam-1* mutants, confirming that centrosome cued polarity is absent in these mutants but can be restored by blocking centrosome movements. We conclude that PAM-1 is a key regulator of centrosome dynamics in the early embryo and regulates anterior–posterior polarity establishment by ensuring close association of the sperm donated centrosome with the posterior cortex.

Materials and methods

Strain maintenance

C. elegans strains were grown and maintained as previously described (Table 1) (Brenner, 1974). *pam-1* mutant strains were grown at the permissive temperature of 15 °C and shifted to the restrictive temperature of 25 °C for at least 5 hours prior to analysis.

RNA interference

Bacteria expressing double stranded RNA for *dhc-1* and *lis-1* were kindly provided by Gönczy (Cockell et al., 2004). Feeding RNAi was performed as described (Kamath et al., 2003), and worms were treated at 25 °C for time points detailed previously (Cockell et al., 2004).

Production of PAM-1 antibodies

Recombinant PAM-1 was expressed in *E. coli*, purified to homogeneity as previously reported (Brooks et al., 2003), and used to raise antibodies in rabbits using standard protocols (Pepceuticals Ltd. Nottingham, U.K.). The antibodies were subsequently purified from rabbit serum using an affinity column constructed by conjugating recombinant PAM-1 to cyanogen bromide-activated Sepharose 4B (Sigma Aldrich) using standard procedures (Harlow and Lane, 1988). The purified antibody detected a single protein band of the expected 100 kDa in western blots of homogenates of mixed staged *C. elegans* (N2 strain) (not shown).

Immunofluorescence and imaging

Antibody staining was performed as described (Lyczak et al., 2006). Primary antibodies used included, DM1A, α-tubulin (Sigma; 1:250), anti-PGL-1 (1:10,000, kindly provided by S. Strome); anti-PAR-2 (1:10, kindly provided by K. Kemphues), and anti-PAM-1 (1:50). Secondary antibodies used were from Jackson Labs and were FITC and rhodomine conjugated rabbit and mouse antibodies. DAPI staining was done with Vectashield. All images of PAM-1 immunostaining were taken with the same settings to show the difference between wild-type and *pam-1* mutant strains.

All imaging was done on a Nikon C1 confocal with EZC1 software. For GFP strains, Z-stack time-lapse images were acquired every 20–40 s. In all images anterior is on the left.

Results

PAM-1 is a cytoplasmic aminopeptidase

In order to determine the localization pattern of PAM-1 in early embryos, we raised polyclonal antibodies against recombinant purified PAM-1. Immunofluorescence studies showed a specific pattern in wild-type embryos which was undetectable in both *pam-1(or282)* and *pam-1(or403)* mutant embryos (Fig. 1). As staining was undetectable in embryos from both alleles of *pam-1* (Fig. 1B), they were used interchangeably during this study. To document the localization pattern, at least 30 embryos were stained at each embryonic stage. PAM-1 is a cytoplasmic aminopeptidase present throughout the cell during early development (Fig. 1A). During meiosis I, PAM-1 localizes to the cytoplasm just under the cell cortex. By meiosis II, this subcortical localization

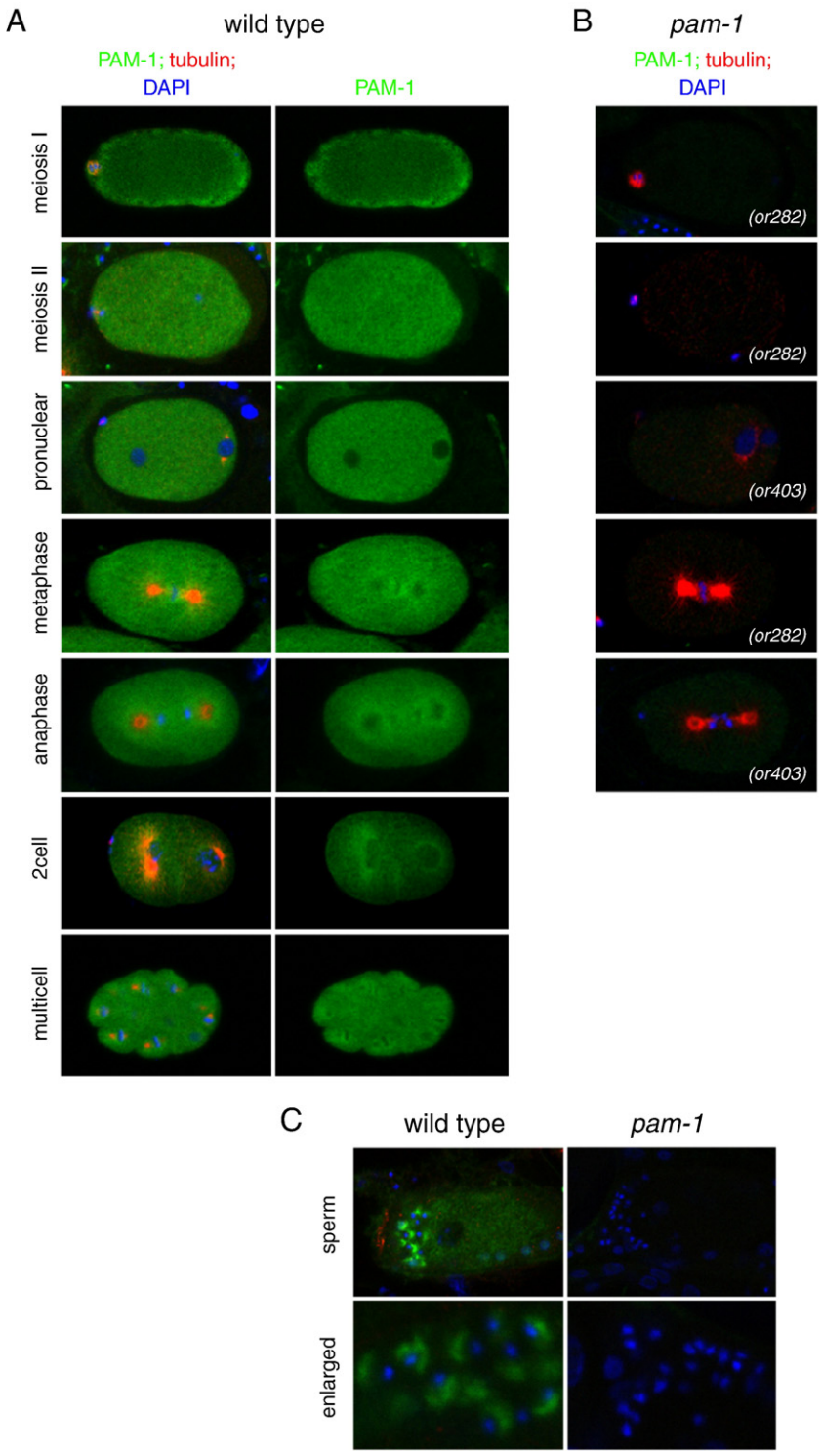

Fig. 1. PAM-1 is localized to the cytoplasm of early embryos. Confocal images of immunostained embryos. (A) In wild-type embryos, PAM-1 localized subcortically and to the cytoplasm during meiosis I. During the remainder of early development, PAM-1 was largely cytoplasmic. However, during mitosis, PAM-1 was found to be concentrated around the mitotic spindle and the mitotic chromosome masses. (B) PAM-1 was found at very low to undetectable levels in all embryos observed from both alleles of *pam-1* used in this study. (C) PAM-1 is also found in the mature spermatids in the spermatheca. An enlarged view shows it is excluded from the chromosomal region of the sperm. This pattern was undetectable in spermatids from *pam-1(or403)* worms. Embryos are approximately 50 μm in length.

extends to the entire cytoplasm where it is seen throughout early development. During the first cell division and beyond, PAM-1 is enriched around mitotic chromosomes, clearly seen in metaphase and anaphase, and near the spindle poles. Previous work suggested that PAM-1 is supplied by the sperm (Lyczak et al., 2006), and indeed we observed PAM-1 expression in the mature spermatids in the spermatheca that was absent in sperm from mutant worms (Fig. 1C).

Anterior and posterior PAR protein domains are not properly established in pam-1 mutant embryos

Our previous work with *pam-1* mutants showed loss of anterior–posterior polarity and mislocalization of the PAR proteins in many embryos. The mislocalization patterns showed some variability between embryos (Lyczak et al., 2006) and was performed with fixed

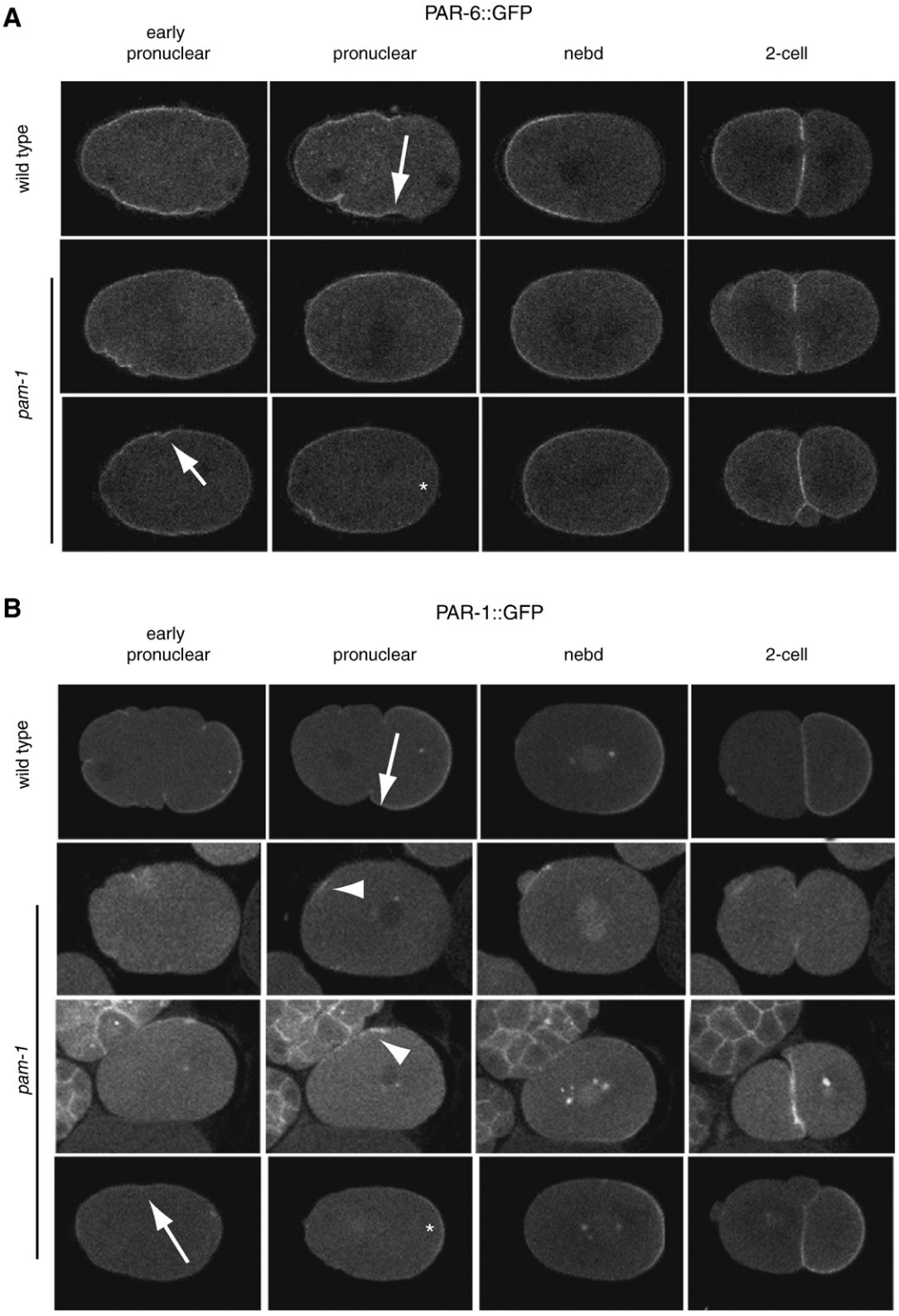

Fig. 2. Anterior and posterior PAR dynamics in *pam-1* embryos. (A) In wild-type embryos, PAR-6 localized around the entire cell cortex until the time of pseudocleavage. A deep pseudocleavage furrow formed (arrow) and PAR-6 cleared from the posterior cortex. By the time of nuclear envelope break-down (nebd), PAR-6 was bright and confined to the anterior half of the embryo. PAR-6 segregated into the anterior daughter after an asymmetric division. In *pam-1* embryos, many embryos exhibited no pseudocleavage, PAR-6 remained around the entire cortex and embryos divided symmetrically. In other *pam-1* embryos, subtle pseudocleavage occurred (arrow) with a short-lived clearing of PAR-6 (*) from the posterior pole. (B) In wild-type embryos PAR-1 localized to the posterior pole around the time of pseudocleavage (arrow) and remained in the posterior half of the embryo through the first mitosis. PAR-1 segregated to the posterior daughter cell after the asymmetric division. In *pam-1* mutant embryos, pseudocleavage was absent or weak (arrow) and PAR-1 was often localized near the polar-body or laterally (arrow heads). However, some embryos exhibited an initially small posterior patch of PAR-1 (*), which expanded prior to the first division. Embryos are approximately 50 μm in length.

specimens. As the dynamics of polarization were not previously examined in *pam-1* mutant embryos, we thought a clear picture of the polarization process in these mutants was missing. We used time-lapse confocal imaging of strains expressing PAR-1 and PAR-6 GFP fusions to further elucidate the polarity defects in these mutants. In wild-type embryos ($n=13$), PAR-6::GFP was initially observed around the entire embryo cortex (Fig. 2A). Initial clearing from the posterior cortex was observed when the sperm centrosome-pronuclear complex abuts the posterior pole, and pseudocleavage occurred. This initial localization was refined and anterior PAR-6::GFP was enriched prior to the first cell division. After division, PAR-6 was observed in the anterior daughter cell AB (Fig. 2A). PAR-1::GFP ($n=17$), was also first observed in the entire cell cortex (Fig. 2B). At the time of pronuclear-centrosome contact with the cortex and PAR-6 clearing from the posterior, PAR-1 became posteriorly localized in wild-type embryos. This localization was enriched and refined prior to the first cell division, and PAR-1::GFP segregated to the posterior daughter, P_1, after division (Fig. 2B).

In *pam-1* mutants, localization of PAR-6 was aberrant and there was some correlation with the pattern of division and PAR-6 localization. Of all *pam-1;* PAR-6::GFP embryos examined, 76% divided symmetrically at the first division (Fig. 2A; $n=19/25$). At the time of division, nearly all these embryos showed PAR-6 localization to the entire cell cortex ($n=18/19$). Of the embryos that divided symmetrically, the majority had PAR-6 around the entire cortex from the time of meiosis and never displayed pseudocleavage (Fig. 2A; $n=14/19$). However, 26% of the embryos that divided symmetrically showed an initial small clearing of PAR-6 from the posterior cortex ($n=5/18$), and some of these embryos also exhibited an attenuated pseudocleavage (Fig. 2A; 3/4). This initial clearing of PAR-6 was in a domain smaller than that seen in wild-type embryos, and PAR-6 was observed to spread back to the posterior cortex prior to formation of the first spindle in most cases (4/5). The remaining *pam-1* embryos divided asymmetrically with anteriorly localized PAR-6 ($n=6/25$; data not shown). Most of these embryos exhibited pseudocleavage that was greatly attenuated from that observed in wild-type, and closer to the posterior pole ($n=5/6$). In half of these asymmetrically dividing embryos, PAR-6 extended toward the posterior and the division resulted in a smaller posterior cell than seen in wild type.

The localization of PAR-1::GFP in *pam-1* mutants varied greatly from embryo to embryo. At the time polarity would be established in wild-type, PAR-1 localization was observed solely at the posterior 39% of the time in *pam-1* embryos (Fig. 2B; $n=9/23$), but in many of these cases, the domain was smaller than in wild-type ($n=4/9$). In 30% of the *pam-1* embryos, PAR-1 was observed on both the putative anterior and posterior pole ($n=7/23$; data not shown). In other embryos, PAR-1 was localized to a lateral patch (Fig. 2B; $n=6/23$), which was sometimes associated with expression near the polar body (Fig. 2B; $n=2/6$), or PAR-1 was absent ($n=1/23$; data not shown). These patterns were dynamic and often changed quickly as the cell entered the first mitosis. In embryos that went on to divide symmetrically, few had posterior PAR-1 ($n=2/10$) but instead had either no PAR-1 at the cortex ($n=4/10$) or other mislocalization patterns (Fig. 2B). In embryos that divided asymmetrically, most had posterior PAR-1 localization by the time of division, in a normal (Fig. 2B; $n=7/13$), or reduced size patch ($n=2/13$). Occasionally, these divisions resulted in a smaller than normal posterior cell ($n=2/13$).

Aberrant centrosome movements in pam-1 mutants require dynein

In wild-type embryos, the centrosomes remain close to the posterior cortex during early development and their association with the cortex is necessary for specifying the posterior pole (Cowan and Hyman, 2004) (Fig. 3; $n=5$). After polarity establishment, the centrosomes, associated with the paternal pronucleus, moved inwards where they met the maternal pronucleus at about 70% egg length. Following this, the pronuclei centrated and the centrosomes rotated to align along the anterior–posterior axis before spindle assembly (Fig. 3). These centrosome movements require the motor protein dynein and its regulator LIS-1 (Cockell et al., 2004; Gönczy et al., 1999). When either dynein heavy chain ($n=12$) or *lis-1* ($n=4$) was inactivated by RNAi, these movements failed and the centrosomes remained in the posterior (Fig. 3).

Previous work in our laboratory showed that the centrosomes in *pam-1* mutant embryos move prematurely and rapidly, spending less time in contact with the posterior cortex (Lyczak et al., 2006). Centrosomes were often observed in the interior of the embryo even before the pronuclei became apparent and close contact with the cortex was not observed (Fig. 3; $n=13/16$). Additionally, centrosomes appeared to robustly nucleate microtubules earlier in *pam-1* mutants. About 46% showed robust sperm asters prior to pronuclear appearance. To look more closely at this difference in embryos with normal pronuclear appearance, we compared sperm asters, in wild-type and *pam-1* embryos expressing tubulin::GFP, at comparable times prior to nuclear envelop breakdown (nebd). Seven minutes prior to nebd in wild-type, sperm asters were small to mid-sized and either touching the posterior cortex or a small distance from the posterior cortex ($n=8$ and Fig. 3). In *pam-1* mutant embryos at the same time point, many embryos had robust asters near the center of the embryo ($n=4/7$ and Fig. 3) while others had small to mid-sized asters similar to wild type ($n=3/7$). Thus, *pam-1* mutant embryos have mispositioned sperm asters that are often larger than wild type at similar times in development.

We sought to test if DHC-1 and LIS-1 were required for the movements of the centrosomes in *pam-1* embryos. When *dhc-1* ($n=14$) or *lis-1* ($n=5$) was inactivated in *pam-1* mutants, we observed that overactive centrosome movements were absent and centrosome positioning mirrored that in *dhc-1* or *lis-1(RNAi)* alone (Fig. 3). Thus we conclude that the overactive centrosome movements observed in *pam-1* mutants require the motor protein dynein.

Blocking centrosome movements restores polarity in pam-1 mutant embryos

pam-1 mutants fail to polarize the anterior–posterior axis, a defect that manifests itself in an absence of pseudocleavage and mislocalization of polarity proteins (Lyczak et al., 2006). Previously, we hypothesized that the premature centrosome movements in *pam-1* mutants may account for the lack of polarity observed in these embryos. To test this hypothesis, we examined pseudocleavage as a measure of initial polarity establishment in embryos depleted of *dhc-1*. When *dhc-1* was inactivated alone, centrosomes failed to leave the posterior cortex and polarity establishment was normal (Fig. 3; $n=12$ and Cockell et al., 2004; Gönczy et al., 1999). In all *dhc-1(RNAi)* embryos observed via time-lapse microscopy, pseudocleavage was clearly evident (Figs. 3–5; $n=35$). In *pam-1* mutant embryos, pseudocleavage almost always failed ($n=54/70$), or was greatly reduced (Figs. 2–5; $n=13/70$). In contrast, when *dhc-1 w*as inactivated by RNAi in *pam-1* mutants, strong pseudocleavage was restored in all embryos (Figs. 3–5; $n=57$). The same result was seen when *lis-1* was inactivated by RNAi (Fig. 3; $n=5$). These data suggest that the centrosome movements in *pam-1* mutants disrupt cortical activity.

As pseudocleavage was restored in *pam-1* mutants in which the centrosomes remained in the posterior, we chose to further characterize the polarization of these embryos by examining localization of cortical proteins. PAR-2 localization was observed through immunofluorescence while PAR-1 and PAR-6 were observed via time-lapse analysis of GFP strains. In wild type, PAR-1($n=17$) and PAR-2 ($n=34$) were observed at the posterior pole, while PAR-6 ($n=13$) was observed at the anterior pole at the pronuclear stage (Figs. 2 and 4). In *pam-1* mutants, only 25% of fixed embryos showed posterior localized PAR-2 ($n=40$) (Fig. 4 and Lyczak et al., 2006). Additionally, as described above, PAR-1 and PAR-6 were frequently mislocalized in *pam-1* mutant embryos (Figs. 2 and 4). When *dhc-1* was inactivated by RNAi in wild type, the normal pattern of localization was retained for PAR-2 ($n=30$), PAR-1 ($n=20$) and PAR-6

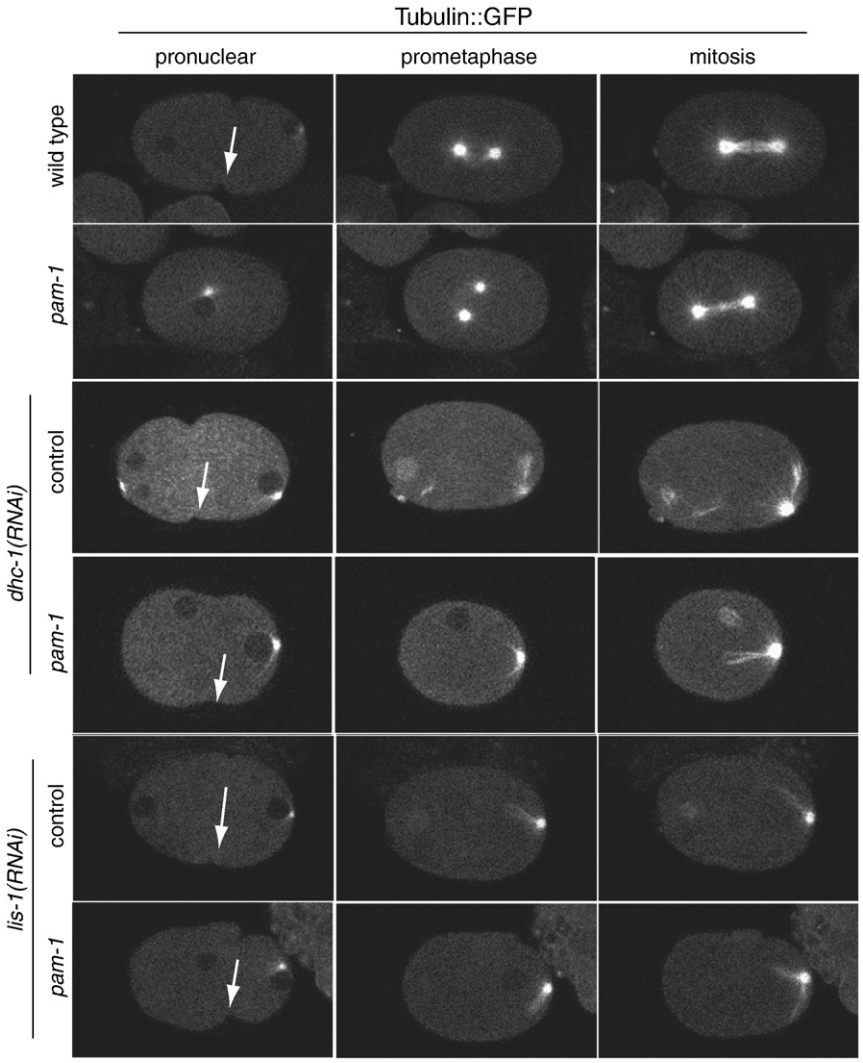

Fig. 3. Inactivation of *dhc-1* or *lis-1* blocked centrosome movements in *pam-1* embryos and restored pseudocleavage. In wild-type, during the pronuclear stage, the centrosomes made contact with the posterior cortex to polarize the axis as observed by the presence of the pseudocleavage furrow (arrow). Centrosomes then moved into the interior of the embryo by prometaphase and the first mitotic spindle was set up. In *pam-1* mutants, pseudocleavage was absent during the pronuclear stage as the centrosomes failed to contact the cortex. Centrosomes often appeared near the center of the embryo, where they remained during prometaphase and spindle assembly. In control embryos depleted of *dhc-1* or *lis-1*, the centrosome remained in the posterior during the pronuclear stage. Polarity was established in these embryos as a pseudocleavage furrow formed normally (arrow). Centrosomes remained at the posterior cortex during prometaphase and mitosis. Likewise, when *pam-1* mutants were depleted of *dhc-1* or *lis-1*, the centrosomes failed to leave the cortex, and pseudocleavage was restored (arrow). Embryos are approximately 50 μm in length.

($n=12$), showing that *dhc-1* is not required for polarity establishment (Fig. 4). When the same was done in *pam-1* mutants, centrosome movement was blocked and localization of the PAR proteins was restored to wild type in all cases (Fig. 4; PAR-2 $n=25$, PAR-1 $n=17$, PAR-6 $n=18$), suggesting the rescue of cortical polarity.

To assess cytoplasmic polarity, we examined germ-line P granule and PIE-1 localization. Each segregates to the posterior pole around the time of pseudocleavage in wild-type embryos (Cheeks et al., 2004; Hird et al., 1996; Strome, 1986; Tenenhaus et al., 1998). Indeed, we observed posterior localization of these markers in wild-type one-cell embryos (Fig. 5; P granules $n=31/34$; PIE-1 $n=6$). Normal localization was infrequent in *pam-1* mutants, with only 24% showing normal P granule localization ($n=62$), and 12.5% showing posterior PIE-1 localization ($n=16$) (Fig. 5 and Lyczak et al., 2006)). Inactivation of *dhc-1* did not disrupt the localization of these cytoplasmic determinants (Fig. 5; P granules $n=39/41$, PIE-1 $n=11$). When *dhc-1* was inactivated in *pam-1* mutants, normal localization of P granules ($n=37$) and PIE-1 ($n=20$) was restored to wild type in all cases (Fig. 5). Thus, both cortical and cytoplasmic polarity is effectively rescued in *pam-1; dhc-1(RNAi)* embryos.

Discussion

Previous work in our laboratory illustrated that the PAM-1 aminopeptidase is necessary for proper axis establishment (Lyczak et al., 2006). Here we have further elucidated the role of PAM-1 in polarity establishment, providing further evidence that the close association between the centrosome and cortex that PAM-1 promotes is crucial for axis establishment. When the microtubule motor DHC-1 is depleted in *pam-1* mutants, premature movement of the centrosome from the cortex is prevented and normal anterior–posterior polarity is established. Thus, PAM-1 is a cytoplasmic aminopeptidase that is required at the earliest step in anterior–posterior axis specification.

PAM-1 localization

PAM-1 is homologous to the human NPEPPS protein. Forms of this protein have been localized to the cell membrane, and others to the cytoplasm (reviewed in Taylor, 1993). From sequence data, it appears that PAM-1 is most closely related to the cytoplasmic form of the protein

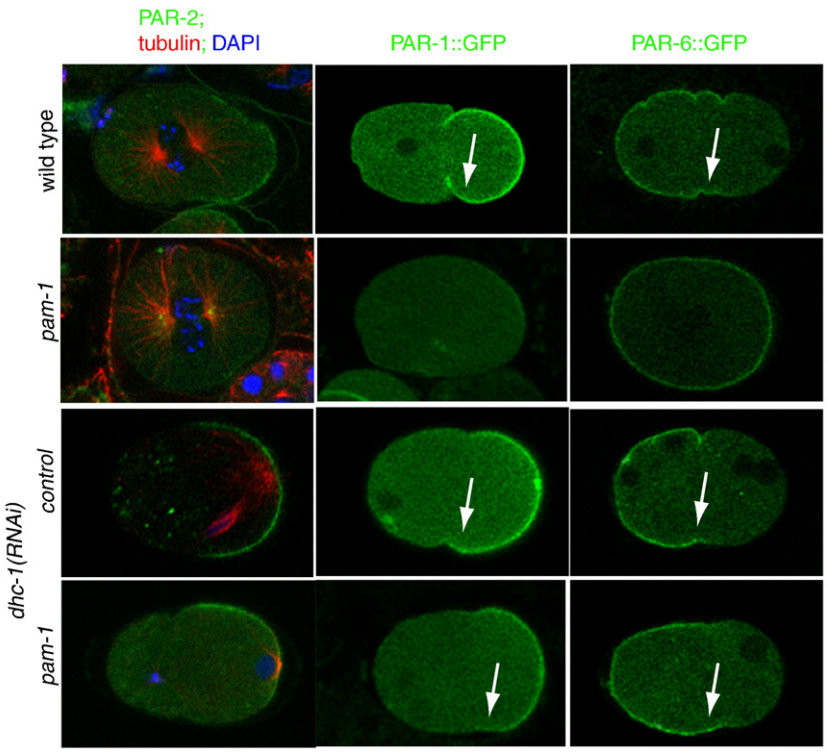

Fig. 4. PAR polarity is restored in *pam-1* embryos when the centrosome contacts the cortex. In wild-type embryos, posterior localization of PAR-2 and PAR-1 and anterior localization of PAR-6 was observed prior to the first mitosis. In *pam-1* mutants, these proteins are often mislocalized at this stage. When *dhc-1* was inactivated by RNAi in controls, normal PAR localization occurred as in wild-type. This was also observed in *pam-1; dhc-1(RNAi)* embryos which exhibited pseudocleavage and wild-type PAR localization. Pseudocleavage is noted by arrows. Embryos are approximately 50 μm in length.

(Brooks et al., 2003). Our localization studies confirm this, clearly showing that PAM-1 is present throughout the cytoplasm of early embryos. Enrichment around the mitotic chromosomes and microtubules suggest that PAM-1 may be playing a role in mitosis. Indeed, work from our lab has noted that *pam-1* mutants often display chromosome segregation defects (Lyczak et al., 2006 and unpublished data). Interestingly, a similar localization pattern was seen in cultured cells expressing the murine homolog (Constam et al., 1995). In our previous genetic studies, we found that mating of *pam-1* females with wild-type males was enough to produce some viable offspring, suggesting that this paternal contribution is sufficient in some cases for early development (Lyczak et al., 2006). In further support of this paternal contribution of PAM-1, we observed localization in mature sperm prior to fertilization. Paternal and maternal contribution of the protein may act to ensure that PAM-1 is present near the posterior pole where it most likely acts in axis polarization.

Defects in polarity establishment

Failure of *pam-1* mutant embryos to polarize along the A–P axis has been shown in past studies (Lyczak et al., 2006). However, while pseudocleavage, an early sign of polarity was absent in nearly all *pam-1* embryos observed, PAR localization, P granule localization and an asymmetric cleavage were still observed in some cases. To better understand this, we examined PAR-1 and PAR-6 localization patterns in living embryos to learn the dynamics of any partial polarization observed. These studies confirmed that the defect in *pam-1* embryos does indeed correspond to the time when the centrosome normally contacts the cortex in wild type. In nearly all *pam-1* embryos, PAR-1 and PAR-6 were not localized properly during this early time point. Confirming past studies that utilized fixed specimens, we observed PAR-6 around the entire cortex during this time. Additionally, we observed no consistent PAR-1 localization pattern. These data are similar to those observed when the centrosome is ablated prior to polarization, or when centrosome function is compromised (Cowan and Hyman, 2004; Hamill et al., 2002; O'Connell et al., 2000). Thus, these data from time-lapse PAR imaging further support the hypothesis that PAM-1 mutants fail to establish centrosome-cued polarity.

While we did observe PAR localization in a subset of embryos, it was often incomplete. For instance, in some embryos, PAR-6 cleared from the posterior or expanded its domain further into the posterior than in wild type. Similarly, some *pam-1* embryos showed smaller posterior patches of PAR-1. These signs of polarization often were seen in embryos that exhibited attenuated pseudocleavage. Occasionally, these initial weak signs of polarity were strengthened and PAR localization looked normal by the time of division. This recovery mimicked embryos in which centrosomes are ablated after the start of polarization (Cowan and Hyman, 2004), suggesting that the centrosomes in these *pam-1* embryos may have briefly contacted the cortex before moving into the interior of the embryo. However, in some embryos, early clearing of PAR-6 or loading of PAR-1 at the posterior was only transient. Thus, it appears that some *pam-1* mutant embryos can partially cue polarity, although this weak cue is not always enough to result in complete polarization, indicating that spreading or maintenance of polarization did not occur. These results are in contrast to previous studies that have suggested that the centrosome is not necessary for these later steps in polarity, but only for the initial polarization cue (Cowan and Hyman, 2004). Further work examining the centrosome dynamics in embryos with different PAR localizations may shed light on this apparent difference.

The levels of PAR proteins in *pam-1* embryos may contribute to the different patterns observed. As has been observed in other studies (Tsai and Ahringer, 2007), polarity defects are slightly different in embryos over-expressing anterior or posterior PAR proteins. Analysis of *pam-1* embryos with normal PAR levels show that they divide symmetrically 59% of the time (Lyczak et al., 2006), while those over-expressing PAR-6::GFP divide symmetrically 76% of the time. In contrast, embryos over-expressing PAR-1::GFP divide symmetrically only 42% of the time. Thus, over-expression of an anterior PAR tips the balance toward a more severe polarity defect, compared to those over-expressing

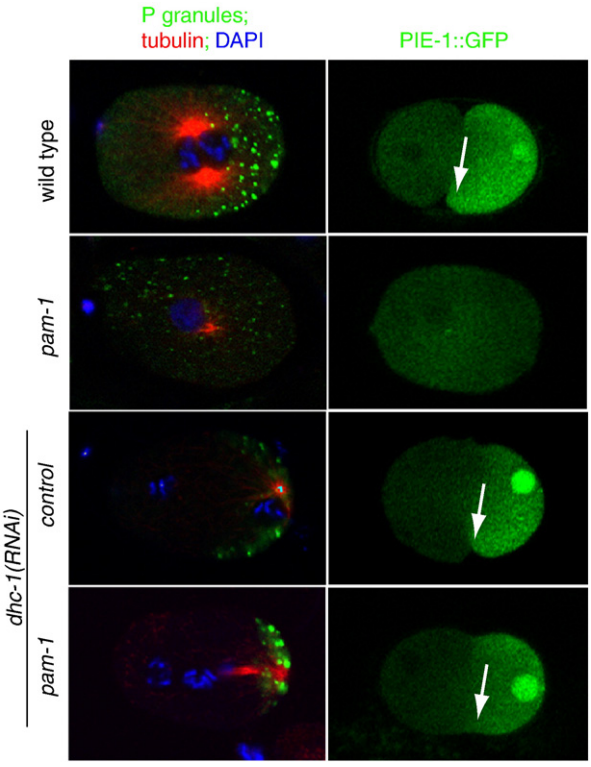

Fig. 5. P granules and PIE-1 localize normally in *pam-1* mutants in which *dhc-1* is inactivated. In wild-type embryos, germline P granules and PIE-1 protein localized to the cytoplasm at the posterior pole prior to the first cell division. Localization of these determinants failed in *pam-1* mutants. Inactivation of *dhc-1* on its own did not affect the normal localization of P granules or PIE-1. When *dhc-1* was inactivated in *pam-1* mutants, both P granules and PIE-1 localized as in wild-type, providing further evidence that polarity was restored in the *pam-1; dhc-1(RNAi)* embryos. Pseudocleavage is marked by arrows. Embryos are approximately 50 μm in length.

a posterior PAR. It is likely that in addition to a lack of centrosome cued polarity, excess PAR-6 further inhibits PAR-1 loading to the posterior, while excess PAR-1 may allow for posterior proteins to load more easily.

The role of the centrosome in cueing polarity

Many studies point to the role of the centrosome in breaking one-cell *C. elegans* symmetry (Cowan and Hyman, 2004; Hamill et al., 2002; O'Connell et al., 2000; Rappleye et al., 2002; Sadler and Shakes, 2000; Sonneville and Gönczy, 2004; Zonies et al., 2010). Our work further supports the idea that close centrosome contact with the cortex is essential for robust cell polarization. All of the defects in *pam-1* mutants are consistent with a defect in the centrosome cue. We previously showed that the centrosomes in *pam-1* mutant embryos move prematurely into the interior of the embryo and spend little time in contact with the cell cortex and hypothesized that the aberrant centrosome dynamics in *pam-1* embryos resulted in the polarity defects observed (Lyczak et al., 2006). If this were true, we predicted that blocking of centrosome movements in *pam-1* embryos would result in restoration of polarity. If instead, PAM-1 was directly required for polarization, anchoring of the centrosomes would not be sufficient to rescue polarity. Our data lends strong support to our hypothesis; when centrosomes were anchored to the cell cortex through inactivation of *dhc-1,* 100% of the *pam-1* embryos exhibited pseudocleavage, normal PAR-1, 2 and 6 polarity and proper P granule and PIE-1 localization. Moreover, it was only by forcing the association of the centrosome with the cortex that the cell was able to polarize. Thus, we show that PAM-1 is dispensable for polarity, if centrosome contact with the cortex is forced. PAM-1 is a crucial component of the polarity machinery acting to ensure centrosome contact with the posterior cortex such that polarity is cued just after the completion of meiosis.

The control of centrosome movement during axis polarization is poorly understood and needs further investigation. While association of the centrosome and the posterior cortex is necessary to cue A–P polarity, the discrete cell cycle stage at which this interaction needs to occur and the length of time of contact that is sufficient are unclear (Cowan and Hyman, 2004). Movement of the centrosomes after sperm aster growth has been studied extensively, and is known to require microtubule motors (Cockell et al., 2004; Gönczy et al., 1999; Kimura and Onami, 2007; Skop and White, 1998). Our results indicate that movements of the centrosomes in *pam-1* mutants require the activity of DHC-1 and its regulator LIS-1, just as normal centrosome movements do (Cockell et al., 2004; Gönczy et al., 1999). However, what stands out in *pam-1* mutants is that this movement is premature. Factors necessary to localize the centrosome to the posterior during the time of polarity establishment and prior to the movements required for centration and spindle assembly have not been identified. PAM-1 is essential to prevent centrosome movements during this time and is the first protein identified that is necessary to ensure the early contact of the centrosome with the cortex.

How then does PAM-1 regulate centrosome contact with the cortex and the dynamics of their movement? While targets of the PAM-1 aminopeptidase remain elusive, it may be that PAM-1 targets motor proteins or their regulators for degradation in the early embryo, thus limiting their activity until after polarity establishment. Another possibility is that PAM-1 regulates an anchoring protein that keeps the centrosome in close proximity to the cortex. Conversely, it may be that PAM-1 acts to limit microtubule nucleation at the sperm asters until after polarization. The premature growth of the sperm asters in *pam-1* mutants may result in the early centrosome movement from the posterior in these embryos. Further work may uncover potential targets of PAM-1 in regulation of the centrosome.

Acknowledgments

We would like to thank P. Gönczy, K. Kemphues, G. Seydoux, and S. Strome for supplying reagents. Thanks to B. Bowerman and R. Kohn for critical reading of the manuscript. This work was funded by NIH-AREA grant 1 R15 GM072523-01 and NSF-RUI grant IOS-0918950. Some of the strains were provided by the *Caenorhabditis elegans* Genetics Center, which is funded by the National Institutes of Health - National Center for Research Resources.

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